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Effects of Elevated O_3 and/or Elevated CO_2 on Lipid Peroxidation and Antioxidant Systems in *Ginkgo biloba* Leaves

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Abstract Four-year-old seedlings of *Ginkgo biloba* were exposed to elevated O_3, elevated CO_2 and elevated O_3 plus elevated CO_2 in open-top chambers (OTCs) to study the responses of antioxidant system in *Ginkgo biloba* leaves. No significant changes in reactive oxygen production and scavenging systems were detected in seedlings exposed to high CO_2. Significant increase in H_2O_2 and MDA content were induced by elevated O_3. The ascorbate content and antioxidative enzymes activity were increased significantly by exposure to high O_3 as well. But the promoted ability in scavenging did not prevent the increase in H_2O_2 content and cell membrane lipid peroxidation. The increase was mitigated by high CO_2 in the combined exposure, but the effect was hardly significant.

Keywords Elevated O_3 and CO_2 · Antioxidant system

Troposphere concentrations of O_3 as well as CO_2 have been increasing rapidly as a result of human activities, and these two major greenhouse gases are likely to increase further, especially in urban areas of China (Wang et al. 2007). Tropospheric O_3 is a dangerous toxicant because it induces production of other reactive oxygen species (ROS) after diffusing into apoplastic solution. ROS were highly phytotoxic and would lead to oxidative stress in plant. Besides stomatal regulations, the resistance of plants to O_3 at cellular level is often correlated with the ability of cells to scavenge ROS by superoxide dismutase, catalase and other antioxidant enzymes in the ascorbate-glutathione cycle (Chernikova et al. 2000). Higher CO_2 may reduce the rate of ROS formation in photosynthesis apparatus for increased ratio of CO_2 to O_2. However, CO_2-driven down-regulation of detoxification system may make plants more susceptible to oxidative stress under higher CO_2 condition (Karnosky 2003). Both CO_2-induced stimulation (Schwanz et al. 1996) and down-regulation (Polle et al. 1993; Kull et al. 1996) of antioxidant systems have been reported. Results from recent years' studies on plant antioxidant systems in elevated O_3 and elevated CO_2 environment are still highly inconsistent though researches on the interactive effects of elevated O_3 and CO_2 on plant antioxidant systems have been started since the early 1980s (Polle et al. 1993; Tausz et al. 2004).

In the present work on *Ginkgo biloba*, which is one of the commonly used street trees for urban greening in Shenyang and other cities, hydrogen peroxide production, malondialdhyde (MDA) accumulation, ascorbic acid content and the specific activity of antioxidant enzymes were determined in leaves to investigate whether elevated CO_2 would ameliorate the oxidative stress in plant exposed to elevated O_3. Under the background of globe climate change, especially the rapidly increased O_3 and CO_2 in urban areas of China, studies on physiological and biochemical response to these two major greenhouse gases will be necessary for urban greening. Further, response of the antioxidant system in *Gingko biloba* leaves to elevated

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O_3 was studied previously (He et al. 2006), and to reveal the interactive effect of the two major greenhouse gases on the antioxidant systems in *Gingko biloba* leaves this work was conducted.

Materials and Methods

The experiment was performed at Shenyang Arboretum of Chinese Academy of Sciences (41°46′N, 123°26′E), located in urban area of Shenyang city. Twenty-four-year-old *Ginkgo biloba* seedlings were transplanted into each chamber in late-March 2006 and fumigated with elevated O_3 (80 ppb) and CO_2 (700 ppm), alone or in combination since 15 June for the whole growing season. Additional O_3 was supplied from 8:00 am to 5:00 pm in sunny days. Additional CO_2 was supplied 24 h each day. Chambers with ambient air (O_3, 40 ppb; CO_2, 390 ppm) were set as control and the gases supply and monitor systems were controlled by computer software, as He et al. (2007) described.

Foliar samples, fully expanded leaves without obvious visual damage symptom, were collected at 9:00 am and plunged into liquid N_2 for later biochemical analysis. To calculate dry weight, parallel samples were dried over night at 80°C.

The concentration of H_2O_2 in leaf tissue was determined according to the method of Mukherjee and Choudhuri (1983) with minor modification. Briefly, 0.5 g of leaf segments was ground into fine powder under liquid nitrogen and then homogenized with pre-cooled acetone (5 mL), and finally centrifuged at 10,000 g and 4°C for 10 min. One half milliliter of the supernatant was mixed with 0.5 mL cooled acetone, 0.1 mL 5% Ti $(SO_4)_2$ and 0.2 mL 19% ammonia. After the precipitate was formed, 1.3 mL reaction mixture was centrifuged at 10,000 g and 4°C for 10 min. The resulting precipitate was dissolved in 5 mL 2 mol L^{-1} H_2SO_4 and the absorbance of Ti^{4+}-H_2O_2 compound was recorded at 415 nm. The H_2O_2 concentration was calculated according to a standard curve of H_2O_2.

Malondialdehyde (MDA) and Ascorbate (ASA) were extracted together with 5 mL of 5% (w/v) trichloroacetic acid at about 0°C. The thiobarbituric acid test was used to assay the MDA content in leaves according to the method of Hodges et al. (1999). The concentration of thiobarbituric acid reactive substances (TBARS) was calculated as MDA equivalents using the extinction coefficient of 155 mmol mol^{-1} cm^{-1} for malondialdehyde and expressed on a dry weight (DW) basis. Ascorbate analysis was performed on a Waters Alliance 2695 HPLC system and detected with a Waters 996 photodiode array detector according to the method of Liu et al. (1994). The column was eluted at 1 mL min^{-1} with 0.5% (w/v) $NH_4H_2PO_4$ solution at pH 2.5. AsA was quantified at its absorption maximum of 254 nm.

Soluble protein and enzymes superoxide dismutase (SOD) and catalase (CAT) was extracted with potassium phosphate buffer (PH 7.8) containing 1 mmol mol^{-1} EDTA and 2% polyvinylpyrrolidone (PVP) on ice. The supernatant was transferred to a new tube and kept at 4°C for immediate analysis. SOD activity was assayed by the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) according to the method of Krivosheeva et al. (1996). CAT activity was determined by the decrease of absorbance of H_2O_2 at 240 nm in 1 min (Aebi and Lester 1984). Soluble protein content was assayed following the method of Song et al. (2004) using bovine serum albumin (BSA) as a standard.

Ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) were extracted together and immediately analyzed as described by Jin et al. (2003) with minor modification. APX activity was determined by monitoring the decrease in A280 (absorption coefficient 2.8 mmol mol^{-1} cm^{-1}) for 1 min in 1 mL of a reaction mixture containing 0.8 mL of 0.5 mmol mol^{-1} ASA in phosphate buffer (pH 7.0), 0.1 mL of 2 mmol mol^{-1} H_2O_2 and 0.1 mL of crude enzyme. For the detection of DHAR activity, the reaction mixture contained phosphate buffer (pH 7.0) 0.7 mL, reduced glutathione (GSH) 50 mmol mol^{-1} 0.1 mL in the phosphate buffer (pH 7.0), 2 mmol mol^{-1} DHA 0.1 mL, and crude enzyme 0.1 mL. The formation of ascorbate was recorded as the increase in A280 for 1 min after the reaction was started. GR activity was determined by following the consumption of NADPH at 340 nm (absorption coefficient 6.2 mmol mol^{-1} cm^{-1}) for 1 min in 1 mL of a reaction mixture containing 0.82 mL of 1 mmol mol^{-1} oxidized glutathione (GSSG), 0.08 mL of 4 mmol mol^{-1} NADPH in phosphate buffer (pH 7.6), and 0.1 mL of enzyme extract.

Ascorbic acid (ASA, sodium salt), dehydroascorbic acid (DHA), glutathione (GSH), glutathione in oxidized form (GSSG), NADPH, PVP and Triton-X100 were all bought from Sigma and the absorption records were all detected with a Shimazu UV-1601 spectrophotometer.The results presented are the means ($n = 3$–6) of all the measurements. Comparisons between means were conducted using the SPSS 10.0 computer package (SPSS Inc. 1999) for all sets of data, by one way ANOVA and the $p = 0.05$ level of error.

Results and Discussion

Both of H_2O_2 and MDA contents were increased significantly by high-ozone exposure for 40 and 80 days (Fig. 1).

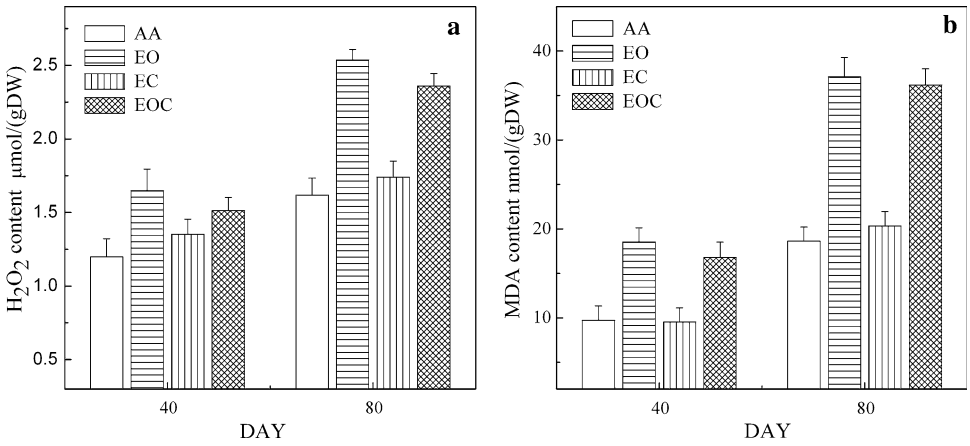

Fig. 1 H_2O_2 content (**a**) and MDA content (**b**) in *Gingko biloba* leaves after 40 and 80 days exposure to elevated O_3 (EO), elevated CO_2 (EC), elevated O_3 + CO_2 (EOC), and ambient air (AA). Values are means ± standard error of 3–6 independent determination

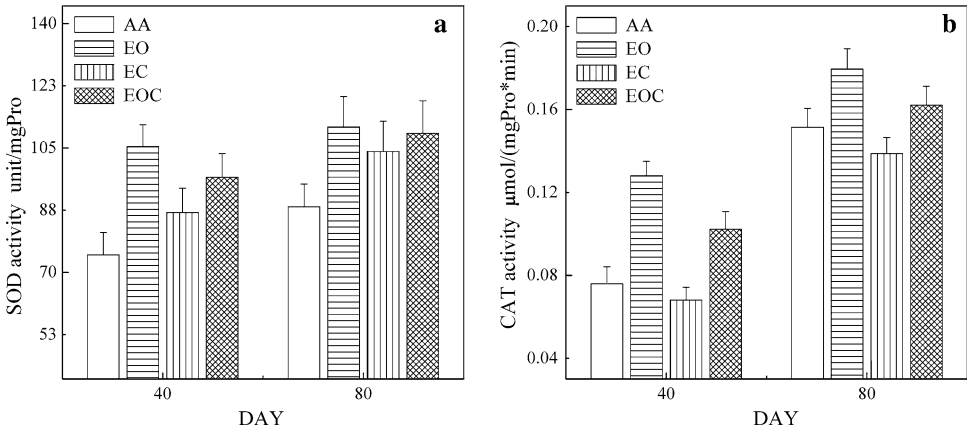

Fig. 2 SOD and CAT activity (**a**, **b**) in *Gingko biloba* leaves after 40 and 80 days exposure to elevated CO_2 (EC), elevated O_3 (EO), elevated O_3 + CO_2 (EOC), and ambient air (AA). Values are means ± standard error of 3–6 independent determination

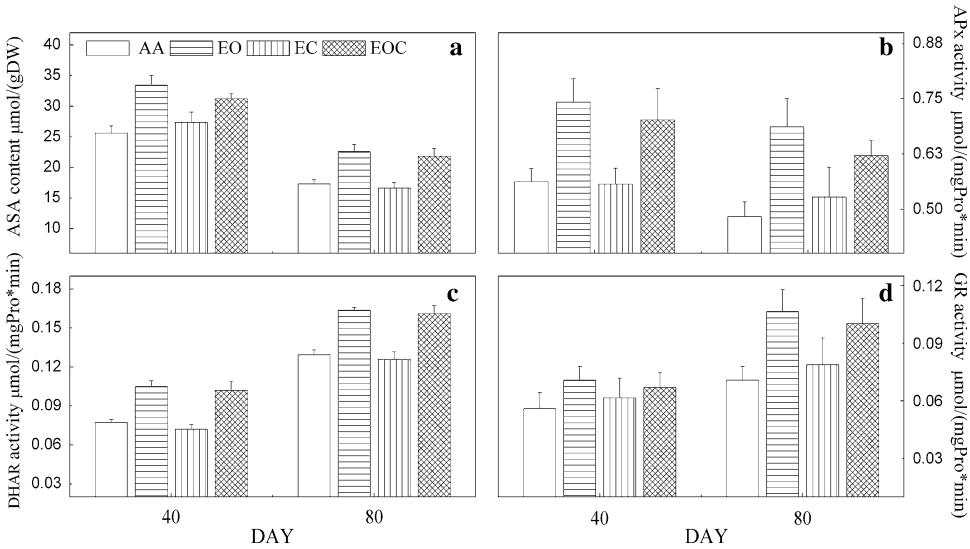

Fig. 3 ASA content (**a**) and the enzyme activities of APX, DHAR and GR in *Gingko biloba* leaves after 40 and 80 days exposure to elevated O_3 (EO), elevated CO_2 (EC), elevated O_3 + CO_2 (EOC) and ambient air (AA). Values are means ± standard error of 3–6 independent determination

The specific activities of enzymes in antioxidant systems (Figs. 2, 3b–d) were also significantly enhanced except for GR on the 40th day (Fig. 3d), and so was the content of ascorbate (Fig. 3a) in *Ginkgo biloba* leaves. It suggested that the increase in activity of antioxidant enzymes was an acclimation effect, but the increase was not sufficient to balance the ROS production, which resulted in the increase in lipid peroxidation as shown by MDA content.

Whether elevated CO_2 would enhance the defense systems in plants is still contradictory. In our research, elevated CO_2 did not significantly change the H_2O_2 and MDA content (Fig. 1). Moreover, the defense system

including the major ROS scavenging enzymes, CAT and APX (Figs. 2b, 3b), and the important small molecular antioxidant ASA (Fig. 3a) did not change significantly either. It may implicated that seedlings exposed to elevated CO_2 produce less ROS and did not need a higher-level antioxidant system. As reported by Karnosky (2003) that, in long term, this could lead to a depressed antioxidant status in plants which would decrease the antioxidant capacity when plants met other stresses.

The effect of simultaneous exposure to both high CO_2 and high O_3 were very similar to exposure to elevated O_3 alone. H_2O_2 and MDA contents of *Ginkgo biloba* leaves (Fig. 1) were still significantly increased, but to a little less extent. Moreover, the specific activity of assayed antioxidant enzymes and ASA content in leaves (Figs. 2, 3) were lower than that exposed to high O_3 alone. It seemed that high CO_2 did not change the response of *Ginkgo biloba* to high O_3 alone. However, increased H_2O_2 and MDA content by high O_3 were mitigated to some extent by high CO_2. Although the mitigating effect may not be statistically significant, the trend was very clearly shown in most measured indexes. These results suggested that high CO_2 perhaps reduced O_3-induced oxidative stress through reducing ROS production (Fig. 1a) which was mainly attributed to decreased O_3 flux into leaf apoplast. As a result, the damage to the cell membrane was alleviated (Fig. 1b) in comparison to exposure of elevated O_3 alone. The lower antioxidant status (Figs. 2, 3) under combined treatment confirmed the lower demand of ROS scavenging in comparison to elevated O_3 alone.

Differences in the response to exposures were discernible between the 40th and 80th day. Higher level of H_2O_2 and MDA content (Fig. 1) on the 80th day perhaps indicated the seedlings' natural senescence and/or the accumulative effect of O_3 exposure with or without elevated CO_2. This view was supported by the higher ASA content (Fig. 3a) on the 40th day. On the 80th day the specific activity of enzymes assayed in Ascorbate-Glutathione cycle was more stimulated by high O_3 with or without high CO_2, especially GR (Fig. 3d), of which was significantly stimulated by 50.6% or 41.8%, respectively. In correspondence, on the 40th day the enhancement of SOD and CAT activity (Fig. 2) by O_3 exposure with or without elevated CO_2 was much stronger than that on the 80th day, 40.7% and 68.9%, 29.1% and 34.7%, respectively in comparison with that under ambient air. It revealed that different compartments of the antioxidant system in *Ginkgo biloba* leaves responded to elevated O_3 or/and CO_2 distinctively at different periods in the growing season. To reveal the defense mechanism of plants against oxidative stresses at cellular level further researches will be needed.

In conclusion, the enhancement of antioxidant system in *Ginkgo biloba* seedlings exposed to elevated O_3 alone was not sufficient to balance the increased H_2O_2 and MDA production. In the air with elevated CO_2 alone, it is not necessary to enhance the defense system against oxidative stress, however, in the case plant exposed to more oxidative environment like elevated O_3, the elevated CO_2 showed an effect in mitigating the oxidative stress.

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